# CYTOPROTECTIVE EFFECTS OF DIETARY FATTY ACIDS ON OXIDATIVE STRESS IN HEPATOCYTES

A Senior Scholars Thesis

by

THOMAS CHARLES RICHARDSON

Submitted to the Office of Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2010

Major: Chemical Engineering

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Associate Dean for Undergraduate Research: Robert C. Webb

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# ABSTRACT

Cytoprotective Effects of Dietary Fatty Acids
On Oxidative Stress in Hepatocytes. (April 2010)

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Non-alcoholic fatty liver disease or NAFLD is characterized by the accumulation of fat in the liver, also known as steatosis. This is one of the most common types of liver diseases and can occur in a range of individuals. Once the liver is affected by steatosis, the liver becomes more susceptible to more serious conditions such as non-alcoholic steatohepatitis, cirrhosis, hepatocellular carcinoma, and liver failure. This research focuses on studying the susceptibility of hepatocytes treated with various common dietary fatty acids to subsequent inflammation and stress. Hepatocytes (HepG2 cell line) were treated with oleic, linoleic and palmitic acid at different concentrations and exposed to the pro-oxidant *tert*-butyl hydroperoxide (*t*-BHP) to induce oxidative stress. The extent of cytoprotection by fatty acid pre-exposure was determined based on the release of Lactate Dehydrogenase (LDH) into the supernatant from dead/dying cells. After exposure, the cells were assayed for various markers of oxidative stress including membrane integrity, lipid peroxidation, and protein carbonylation. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed on the

carbonylated proteins followed by commassie blue staining for visualization and analysis. In general, samples with higher carbonyl content showed more intact proteins on gels. Our analysis identified four specific bands which were present in samples from cells untreated with *t*-BHP, as well as fatty acid treated cells. Such bands are most likely proteins that are susceptible to degradation upon carbonylation. Identification of these proteins by mass spectrometry will provide insights into the proteins susceptible to oxidative stress during steatosis, as well as serve as likely biomarkers for complications that arise from steatosis.

# ACKNOWLEDGMENTS

I would first like to thank my research advisor, Dr. Arul Jayaraman. Dr. Jayaraman first inspired me to seek more out my undergraduate career, which eventually led me to pursue graduate school. His willingness to teach undergraduates about research and what academic research is about is commendable.

I would also like to thank my graduate student research advisor, Billy Newton. Billy spent countless hours helping me and teaching me, of which I can never express enough gratitude. He was and is, always open to answering my never ending questions.

Finally, I would like to think my friends and family who supported me throughout my research work.

# NOMENCLATURE

| | |
|---|---|
| ABC | Ammonium Bicarbonate |
| AU | Arbitrary Florescence Units |
| BME | Beta Mercapto Ethylene |
| HepG2 | Hepatocyte Cell Line |
| LDH | Lactate Dehydrogenase |
| NAFLD | Non-alcoholic Fatty Liver Disease |
| PBS | Phosphate Buffered Saline |
| ROS | Reactive Oxygen Species |
| SDS-PAGE | Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis |
| SDS | Sodium Dodecyl Sulfate |
| *t*-BHP | *tert*-Butyl Hydroperoxide |

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# CHAPTER I

# INTRODUCTION

## Background

Non-alcoholic fatty liver disease or NAFLD is characterized by the accumulation of fat in the liver, also known as steatosis. This is one of the most common types of liver diseases and can occur in a range of individuals. Once the liver is affected by steatosis, the liver becomes more susceptible to more serious conditions such as non-alcoholic steatohepatitis, cirrhosis, hepatocellular carcinoma, and liver failure[1]. There are many factors that effect to onset and progression of this disorder, which include genetic, dietary, viral, and/or behavioral. My research focuses on studying the effects by treating hepatocytes with various common dietary fatty acids.

An excess of fatty acids is generally thought of as bad as it leads to further complications (i.e., the two-hit hypothesis), but it has been shown that low levels of oleic acid can actually be beneficial and induce a cytoprotective effect against lipid peroxidation[2,3].

## Effects of fatty acids

By studying the effects of exposing liver cells in culture to dietary fatty acids we hope to understand more about the mechanisms underlying fatty-liver disease. Several groups have developed in-vitro steatotic models; and a survey of recent findings reveals that individual fatty acids have dissimilar effects on steatosis that are time and concentration

This thesis follows the style of the *Journal of Proteome Research*.

dependant[3-5]. Moderate to high concentrations of palmitic acid has been found to be toxic to HepG2 cells and shown to activate pro-apoptotic pathways[4]. On the other hand, Damelin et al. have recently shown that low to moderate doses of oleic acid actually increase cellular resistance to pro-oxidant challenge[3]. This protective effect was observed only at low concentrations and was not detected with other unsaturated fatty acids. Low doses of linoleic acid have been found to produce greater cell death and more lipid peroxidation than palmitic or oleic acid[5].

**Reactive oxygen species and carbonylation**

Reactive oxygen species (ROS) are ubiquitous as normal metabolism results in the generation of different ROS, such as superoxide ions[6]. The ROS generated are normally cleared away by the cellular anti-oxidant machinery, and a state of oxidative stress arises when the rate of generation (and deleterious effects) of reactive oxygen species is greater than their rate of removal by cellular antioxidant and repair mechanisms[6]. Carbonylation is the non-enzymatic addition of a carbonyl to a protein resulting from the effects of ROS and is a common bio-marker for oxidative stress[7]. It is possible that cells treated with different dietary fatty acids have different levels of basal oxidative stress, and therefore, different susceptibility to subsequent pro-oxidant challenge. We believe that loading with different dietary fatty acids leads to different levels of oxidative damage and subsequent carbonylation. The amount, pattern and identification of carbonylated proteins will provide insights into how dietary fatty acids modulate cellular response to oxidative stress.

**Project overview and summary**

This research aims to test the effects of fatty acids in hepatocytes and show they are correlated to the levels of protein carbonylation upon oxidative challenge. We are studying the effects of dietary fatty acids on hepatocytes (HepG2 cell line) and whether these different fatty acids make the cells more vulnerable or resistant to oxidative stress. The goal is to gain an understanding of possible mechanisms behind the progression of steatosis to more serious conditions such as non-alcoholic fatty-liver disease. We will load HepG2 cells with oleic, linoleic and palmitic acid at different concentrations and expose them to the pro-oxidant *tert*-butyl hydroperoxide (*t*-BHP) to induce oxidative stress. After exposure, the cells will be assayed for various markers of oxidative stress including membrane integrity, lipid peroxidation, and protein carbonylation. We expect linoleic acid loaded cells to be the most susceptible to oxidative stress and have the highest degree of protein carbonylation.

First, we will experimentally determine the correct dose for each of the fatty acids to load the HepG2 cells with. In order to accomplish this the HepG2 cells are seeded in well plates and exposed to individual fatty acids at different (4 – 6) concentrations ranging from 0 – 1.0 mM for a period of 24-48 h. At the end of the exposure, culture supernatants are collected and assayed for release of the intracellular enzyme LDH using the Cytotox-One LDH cytotoxicity assay (Promega, Madison, Wisconson). This will be done for each of the fatty acids in order to determine the range of concentrations over which the experiments are to be carried out. HepG2 cells will be loaded with the

optimum concentration of each fatty acid, followed by exposure to the pro-oxidant molecute *t*-BHP for 8-12 hours. The extent of cytoprotection by fatty acid pre-exposure will be determined based on the release of LDH into the supernatant from dead/dying cells. Affinity chromatography will be performed to isolate carbonylated proteins from the supernatant of exposed cells. The cells will be treated with biotin hydrazide, which binds to aldehyde group of the carbonylated protein and passed though a column[8] containing avidin linked beads which bind the biotinylated carbonylated protiens[8]. After washing, the proteins will be eluted from the column[8]. SDS-PAGE will be performed on the carbonylated proteins followed by protein staining, which separates the proteins by size and allows for visual comparison of the proteins from different samples. Samples containing more carbonylated protein will produce gel bands of greater intensity. It is possible that bands appear in sample from cells treated with one fatty acid, but not another. Such bands are likely proteins that are susceptible to carbonylation. These gel bands will be excised, and the proteins they contain can be eluted and identified by mass spectrometry analysis (at the Laboratory of Biological Mass Spectrometry, TAMU).

It is known that treating hepatocytes with low levels of oleic acid produces a protective effect from oxidative stress, but other fatty acids do not have this same effect. As mentioned before we are exposing the cells to different fatty acids, which should lead to different levels of oxidative stress. We expect cells treated with palmitic acid will experience the highest cell death. In addition, cell treated with high concentrations of

fatty acid followed by high concentration of *t*-BHP should exhibit the highest degree of carbonylation.

# CHAPTER II

# MATERIALS AND METHODS

## Reagents and supplies

All reagents and supplies for cell culture, cell harvesting, and chromatography materials were purchased from Thermo Fisher (Hampton, NH) unless otherwise noted. Cells culture media was purchased from Hyclone (Logan, UT) and all fatty acids were purchased from MP Biomedicals (Solon, OH). Cytotox-One LDH cytotoxicity assay was purchased form Promega (Madison, WI). Protease Inhibitor cocktail was purchased from Sigma (St. Louis, MO) and SDS-PAGE materials were purchased form Bio Rad (Philadelphia, PA).

## Cell culture

The HepG2 (ATCC # HB-8065) cells were maintained in Modified Eagle's minimal essential medium with Earle's salts (MEM-EBSS) with high glucose (Hyclone) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 U/mL streptomycin in a humidified atmosphere of 95% air, 5% CO2 at 377C. All the experiments were carried out while the cells were in exponential growth phase. The cells were passed from the culture flasks at 70-80% confluence with 0.25% trypsin in PBS with 0.02% EDTA, and resuspended in media containing 10% FBS,

**Fatty acid loading**

HepG2 cells were seeded in a 24-well plate ($1\times10^5$ cells/well). Upon reaching 60 – 70 % confluency the cells were treated with the fatty acid (oleic, palmitic, or linoleic), in concentrations ranging from 0 – 1.0 mM, for a period of 48 hours. After completion of the fatty acid exposure, the culture supernatants were collected and replaced with media. Supernatants were stored at -80 ℃.

**Pro-oxidant challenge**

Following fatty acid exposure as described previously, the cells were exposed to media containing 0.50 mM *tert*-butyl Hydroperoxide (*t*-BHP) for a period of 8 hours after which culture supernatants were collected and stored at -80 ℃.

**LDH cytotoxicity assay**

Culture supernatants collected after fatty acid exposure and *t*-BHP challenge were assayed in triplicate for release of the intracellular enzyme LDH using the Cytotox-One LDH cytotoxicity assay. The supernatants were assayed using the 100 µl format with a 10 minute exposure period, according to manufacturer's instructions.

**Carbonylated protein isolation**

After fatty acid and *t*-BHP exposure, approximately $6\times10^6$ cells (cells from 3 wells of a six well plate) were harvested using trypsin, and pelleted in 500 µl PBS and stored at -80 ℃ until further use.

The following chemicals were added to each of the cell pellets: Protease Inhibitor cocktail to a concentration of 0.3 % v/v, biotin hydrazide (Thermo Fisher) to a concentration of 8 mM, and SDS to a concentration of 0.5 % wt/v SDS. Each cell pellet mixture was sonicated using a Model 60 sonic dismembrator (Fisher Scientific) on ice at 4 W for 15 sec; this is the whole cell lysate. The mixture was incubated for 60 min and then sodium cyanoborohydride was added to a concentration of 10 mM to reduce hydrazone bonds. The samples were dialyzed to remove excess reagents using Slide-A-Lyzer Cassettes (7.5 k MWCO, Thermo Fisher). The samples were incubated for 24 hours at 4 °C, changing the dialysis buffer every 8 hours. The protein content of the dialyzed sample was assayed using the Better than Bradford 660 nm dye (Thermo Fisher) according to the manufacturer's instructions.

Affinity purification was performed using batch style processing in a chromatography column. Each column was filled with 400 µl of 50 % wt. supported monomeric avidin slurry (Thermo Fisher Product No. 20228) to obtain approximately 200 mg of monomeric avidin beads in the column. One ml of Eluting and Blocking buffer (2 mM D-Biotin in 25 mM ammonium bicarbonate (ABC) pH 7.6) was added to each column and centrifuged in order to bind non-revervisble sites. Biotin is removed by adding 1 ml of stripping buffer (100mM Glycine pH 2.8) to the column and centrifuging. Each of the samples were added to a column and incubated on rocker for 1 hour to capture biotinylated carbonlyated proteins. The samples were centrifuged and the supernatants were collected; this is the Load sample. The avidin Beads were washed and centrifuged

3 times with 1 ml of ABC. Biotinylated carbonlyated proteins were eluted by adding 300 μl Eluting and Blocking buffer to the column, centrifuged and collected in a tube for each sample. Each of the Load and Elute samples were assayed by the Bradford assay using the Better than Bradford dye. The Elute samples were lyophilized before SDS-PAGE.

**SDS-PAGE**

Each lyophilized carbonylated protein sample was rehydrated using Laemili buffer with 0.8 % v/v BME. To run the gel, the Mini-Protean 2 Electrophoresis Chamber (Bio Rad) and a precast wide range 10% gel (Bio Rad) were used. The voltage and running time were set according to manufacturing instruction. After electrophoresis, the gel was rinsed with DI water and fixed with a solution containing 20% methanol and 7% acetic acid. The gel was stained with GelCode Blue Stain Reagent (Thermo Fisher) overnight and then destained in DI water, according to manufacturing instructions. After staining, the gel was imaged with a Versa Doc 3000 imaging system (Bio Rad) under setting for Coomassie stained gels.

# CHAPTER III

# RESULTS

To determine whether each of our different fatty acids render the cells more vulnerable or resistant to oxidative stress, we first developed a dose response curve in order to choose a low and high fatty acid concentration to conduct our experiment. The dose response curve is a representation of how the cells react to a range of fatty acid concentrations. Carbonylated protein isolation was performed at low and high fatty acid concentration each with plus and minus *t*-BHP challenge. SDS-PAGE was performed with samples from oleic and palmitic acid. The results obtained from each of these experiments are presented below.

## LDH cytotoxicity assay

### *Fatty acid loading*

Figure 1 represents the data obtained from the oleic fatty acid dose response curve. The purpose of the dose response curve is to observe the effect of the fatty acid over a range of concentrations to determine optimum experimental conditions.

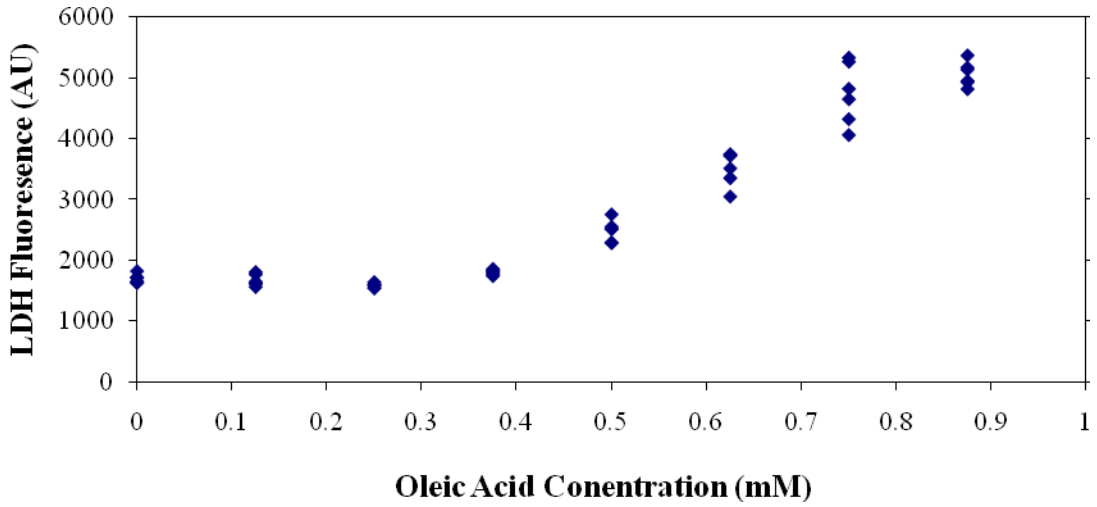


**Figure 1**. Oleic acid dose response curve. HepG2 wells were treated with a range of oleic acid concentrations for a period of 48 hours. The collected supernatants were assayed for LDH release.

This plot clearly shows that less LDH is released in the range of 0.15 – 0.25 mM oleic acid concentration as compared to non-fatty acid loaded cells. After this range the LDH release increased, thus cell death increased.

Figure 2 represents the data obtained for both the linoleic and palmitic acid dose responses.

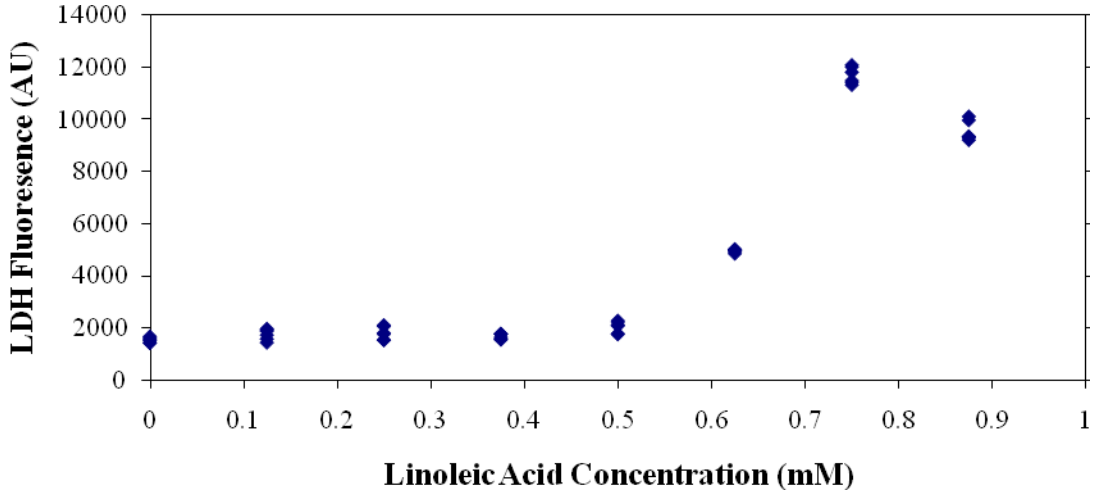


**Figure 2**. Linoleic acid dose response curve. HepG2 wells were treated with a range of linoleic acid concentrations for a period of 48 hours. The collected supernatants were assayed for LDH release.

The LDH release for linoleic acid treated cells stays fairly constant until approximately 0.4 mM linoleic acid, at with point the cells death increases.

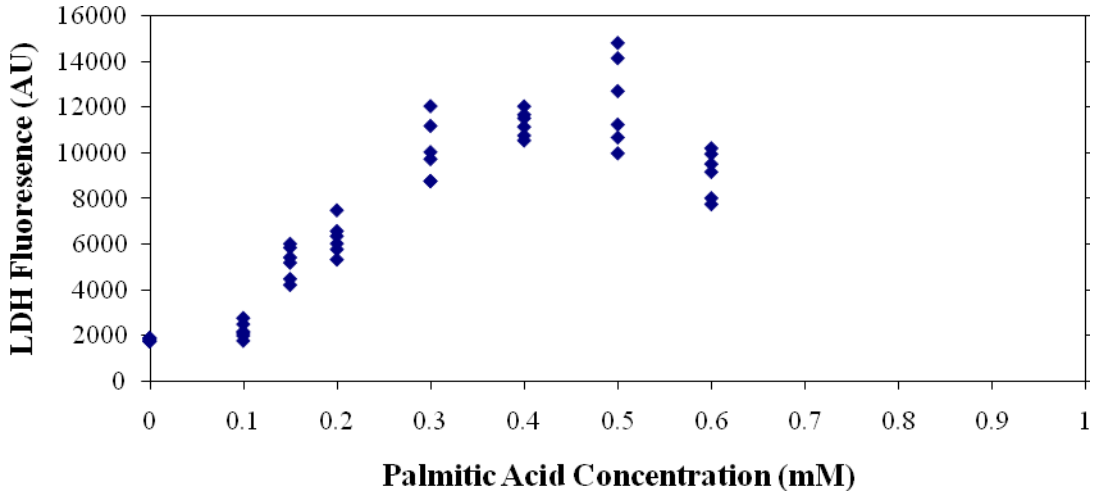


**Figure 3**. Palmitic acid dose response curve. HepG2 wells were treated with a range of palmitic acid concentrations for a period of 48 hours. The collected supernatants were assayed for LDH release.

Figure 3 show that the palmitic acid treated cells immediately become susceptible to cell death and LDH release increases immediately. For each of the linoleic and palmitic acid dose response curves the LDH release decreases at higher concentration due cell death.

*Pro-oxidant challenge*

Figures 4 and 5 show the dose response curves for oleic and linoleic acid treated cells that have been exposed to 0.50 mM *t*-BHP for a period of 8 hours.

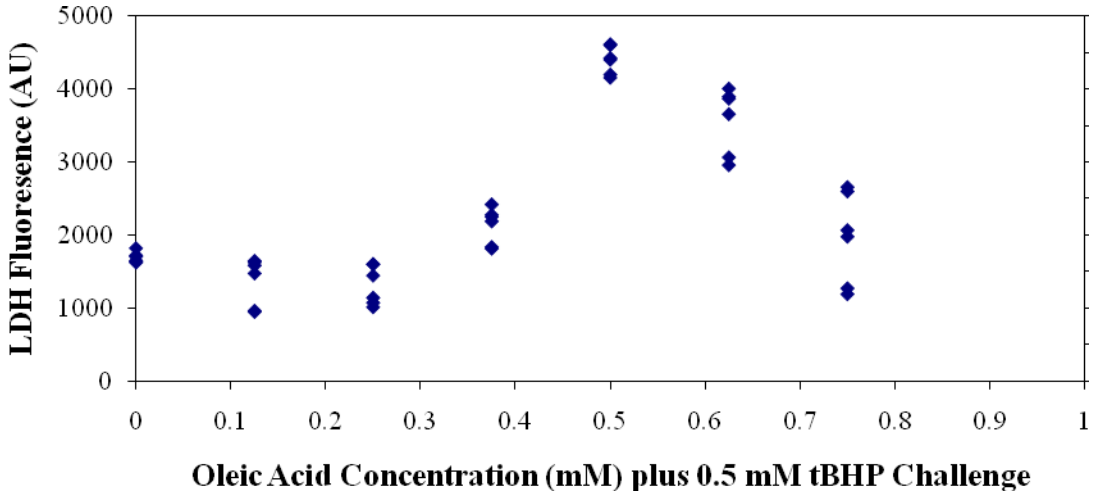


**Figure 4**. HepG2 cells were treated with a range of oleic acid concentrations for a period of 48 hours and the supernatants where collected. After fatty acid exposure, cells were treated with 0.5 mM *t*-BHP for a period of 8 hours and the supernatants were collect. The collected supernatants were assayed for LDH release.

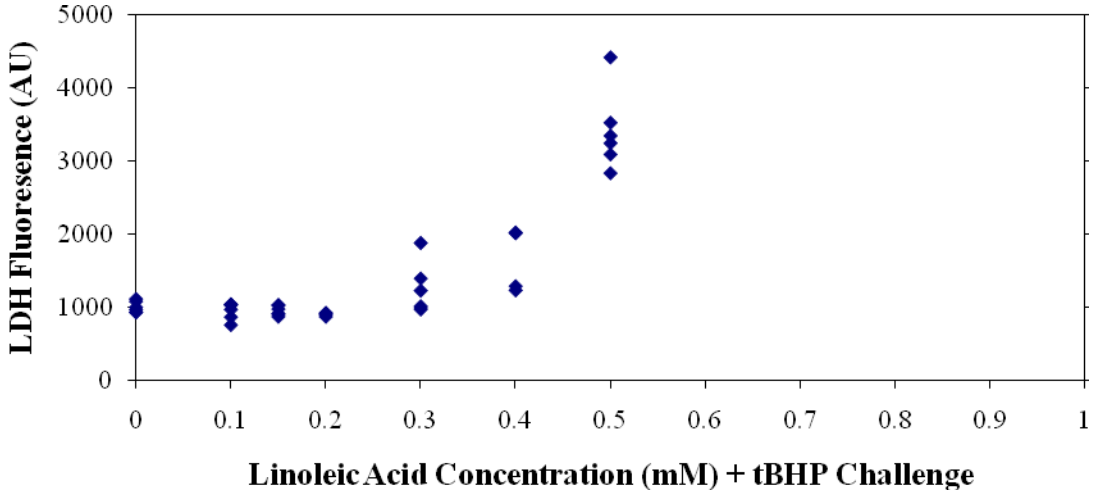


**Figure 5**. HepG2 cells were treated with a range of linoleic acid concentrations for a period of 48 hours and the supernatants where collected. After fatty acid exposure, cells were treated with 0.5 mM *t*-BHP for a period of 8 hours and the supernatants were collect. The collected supernatants were assayed for LDH release.

For the palmitic acid followed by *t*-BHP exposure, the dose response data is not represented well in a plot. The following table shows the erratic LDH response the palmic acid +*t*-BHP experiment yielded. The palmitic acid followed by *t*-BHP exposure

experiment produced a high degree of cell death resulting in poor LDH assay results. The following plot shows no noticeable trends.

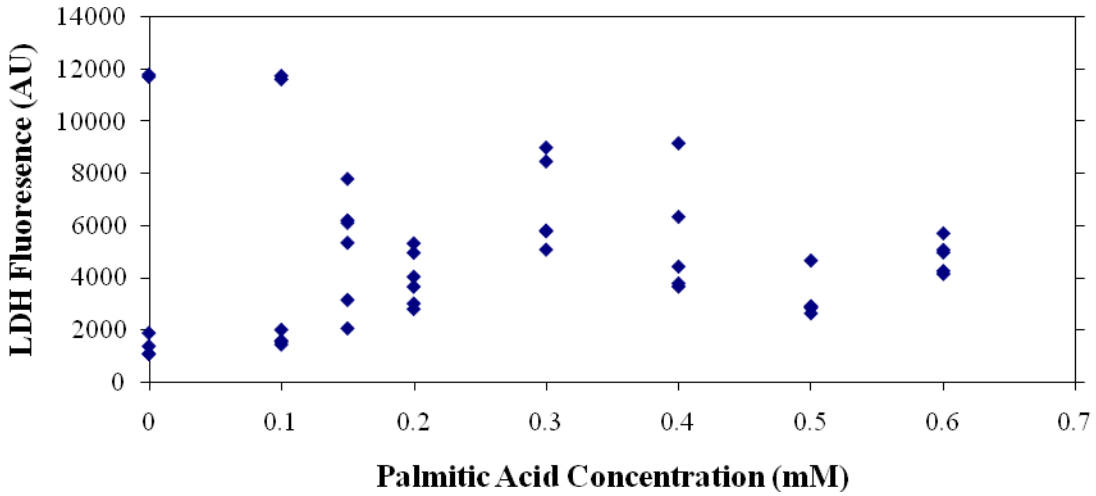


**Figure 6.** HepG2 cells were treated with a range of palmitic acid concnetrations for a period of 48 hours and the supernatants where collected. After fatty acid exposure, cells were treated with 0.5 mM tBHP for a period of 8 hours and the supernatants were collect. The collected supernatants were assayed for LDH release.

**Protein isolation/SDS-PAGE**

Once optimal low and high fatty acid concentrations were determined from the dose response curves, carbonylated protein isolation was performed on the samples by the chromatographic procedures described in Chapter II. SDS-PAGE was performed on the isolated proteins samples as can be seen below. In Figure 6, several bands appear in the fatty acid treated samples which are not readily apparent in other lanes. Another identifiable band appears in both the untreated control and oleic acid only treated sample, and not readily apparent in other lanes. In Figure 7, a band appears in both the untreated control and oleic acid only treated sample that appears much darker than in the other lanes. Figure 8 show similar results for palmitic acid, as shown below.

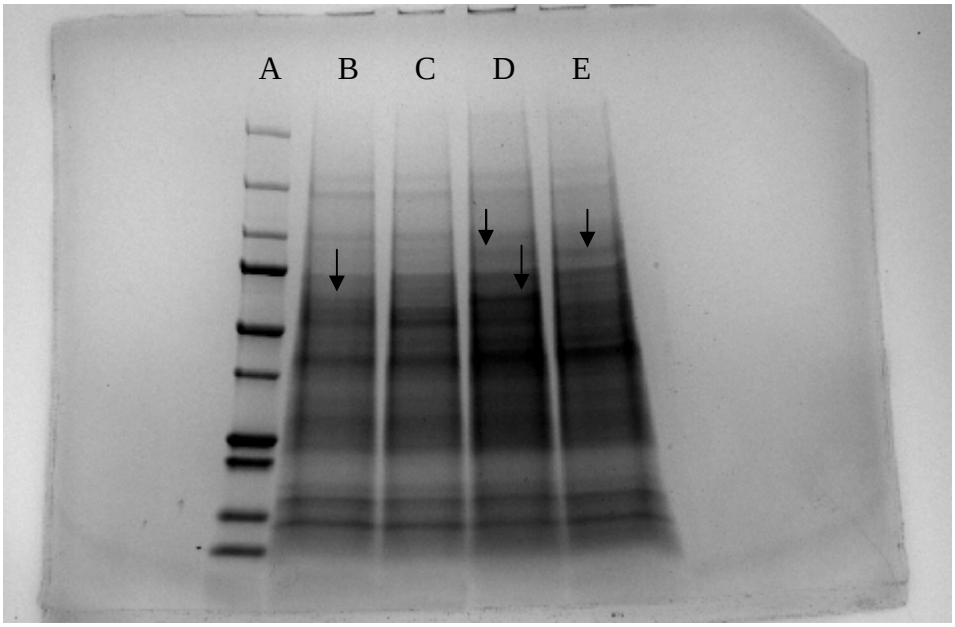


**Figure 7**. Oleic Acid: Carbonylated proteins isolated from untreated and treated HepG2 cell extract. Each lane was loaded with 1200 mg/ml from each protein sample. A) Ladder, B) Control, no fatty acid or tBHP treatment, C) Cells treated with 0.50 mM tBHP for 8 hoursD) Cells treated with 0.15 mM oleic acid for 48 hours, E) Cells treated with 0.15 mM oleic acid for 48 hours, followed by 8 hr exposure to 0.50 mM tBHP.

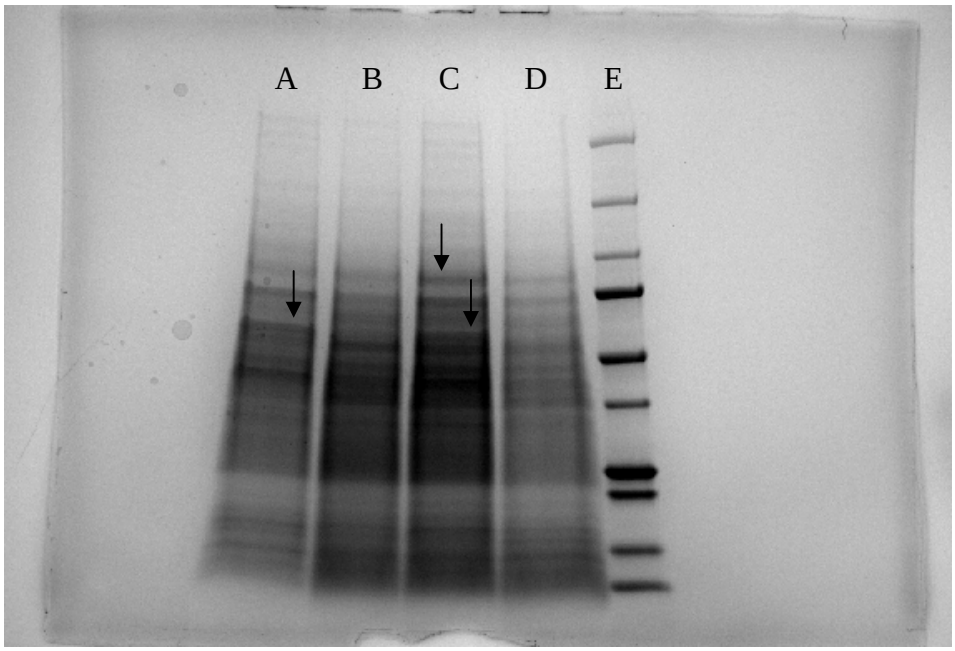


**Figure 8**. Palmitic Acid: Carbonylated proteins isolated from untreated and treated HepG2 cell extract. Each lane was loaded with 1200 mg/ml from each protein sample. A) Control, no fatty acid or *t*BHP treatment, B) Cells treated with 0.50 mM tBHP for hours C) Cells treated with 0.15 mM palmitic acid for 48 hours, D) Cells treated with 0.15 mM palmitc acid for 48 hours, followed by 8 hr exposure to 0.50 mM tBHP. E) Ladder

In general, the LDH assay proved to be the most problematic when trying to optimize experimental conditions. Due to the high degree of variability in the results, the LDH toxicity assay had to be repeated multiple times for each of the three fatty acids to generate acceptable results and off-set the effects of cell death. In addition, a control plate that had no fatty acid or *t*-BHP treatment also resulted in a large standard deviation. We believe this was due to large cell detachment from the plate due to the natural variability of these cell populations. When larger groups of cells detach and lyse, a significant quantity of LDH is released into the supernatant than what would normally be released.

The doubling time of HepG2 cells is 48 hours so there should be significant cell growth during the fatty acid exposure. However, at 0.5 mM fatty acid concentration and higher, significant cell death is observed by the number of floating cells, and far fewer cells are left attached to the plate at the time of supernatant collections, as compared to the low concentration and untreated cells. This results in less LDH released at high fatty acid concentration, which and be seen in the fatty acid dose response curves. Once low and high fatty acid concentrations were determined, we were able to proceed with the rest of the experiments (carbonylated protein isolation and SDS-PAGE) without any significant difficulties.

# CHAPTER IV

# CONCLUSIONS

We developed an experimental model for investigating the effects of oxidative stress in steatotic hepatocytes. We isolated carbonylated proteins as a measure of protein modifications arising due to oxidative stress. This provided insight into how different dietary fatty acids affect oxidative stress by altering levels of protein carbonylation, and in turn how they can affect the susceptibility of the liver to subsequent complications (e.g., infections). The results obtained in the Chapter III follow what we hypothesized in Chapter I with a few exceptions. We expected cells treated with palmitic acid to reveal the highest cell death, and cells treated with high concentrations of fatty acid followed by high concentration of *t*-BHP to exhibit the highest degree of carbonylation.

We determined that oleic acid treated cells produce lower levels of cell death upon *t*-BHP exposure compared to untreated cells, confirming results found by Damelin et al[3]. It was expected that cells treated with unsaturated fatty acid treated cells produce more carbonylation, as unsaturated fatty acids are more vulnerable to lipid oxidation. However in light of the cytoprotective effect of oleic acid, it was unclear what the effect of oleic acid would be on protein carbonylation. Oleic and palmitic acid treated cells produce higher levels of carbonylation compared to non-fatty acid treated cells as shown by SDS-PAGE.

Surprisingly, in both the oleic and palmitic gels, samples from cells not exposed to *t*-BHP reveal higher levels of carbonylation compared to samples from cells exposed to *t*-BHP suggesting tBHP induces cellular mitigation or removal of carbonylated proteins. We expected that the pro-oxidant challenge would reveal higher levels of protein carbonylation.

Cellular response to fatty acid induced carbonlyation may be part of a cytoprotective effect (oleic acid) or increased toxicity (palmitic acid). Oleic acid treated cells have less carbonylation than palmitic acid treated cells suggesting enhanced mitigation of oxidative stress. Further investigation is warranted, to determine if the mechanisms of oleic and palmitic acid induced carbonylation are related, and if palmitic acid induced carbonylation is the major cause of cytotoxicity. Future work will focus on excising the prominent bands marked in the gel results and perform mass spectrometry to identify proteins with increased expression levels and/or more susceptible to protein carbonylation.

# REFERENCES

(1) Kenichiro, Y. Nutritional investigation of non-obese patients with non-alcoholic fatty liver disease: The significance of dietary cholesterol. *Scandinavian J. Gastroenterology.* **2009**, *44*, 471-477

(2) Day CP, James OF. Steatohepatitis: A tale of two‘‘hits’’? *Gastroenterology.* **1998,** *114*, 842–845.

(3) Damelin, L.H. Fat-loaded HepG2 spheroids exhibit enhanced protection from Pro-oxidant and cytokine induced damage. *J Cell Biochem*. **2007**, *101*(3), 723-34.

(4) Gomez-Lechon, M.J. A human hepatocellular in vitro model to investigate steatosis. *Chem Biol Interact*, **2007**, *165*(2), 106-16.

(5) Sung, M. Differential effects of dietary fatty acids on the regulation of CYP2E1 and protein kinase C in human hepatoma HepG2 cells. *J Med Food*, **2004**, *7*(2), 197-203.

(6) Reiter, R.J. Actions of melatonin in the reduction of oxidative stress. A review. *J Biomed Sci*, **2000**, *7*(6), 444-58.

(7) Dalle-Donne, I. Protein carbonylation, cellular dysfunction, and disease progression. *J Cell Mol Med*, **2006**, *10*(2), 389-406.

(8) Newton, B.W. Liver proteome analysis in a rodent model of alcoholic steatosis. *J of Proteome Research*, **2009**, *8*, 1663-1671

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